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Developmental toxicity of plastic leachates on the sea urchin *Paracentrotus lividus*☆

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ABSTRACT

Microplastic pollution has become ubiquitous, affecting a wide variety of biota. Although microplastics are known to alter the development of a range of marine invertebrates, no studies provide a detailed morphological characterisation of the developmental defects. Likewise, the developmental toxicity of chemicals leached from plastic particles is understudied. The consequences of these developmental effects are likely underestimated, and the effects on ecosystems are unknown. Using the sea urchin *Paracentrotus lividus* as a model, we studied the effects of leachates of three forms of plastic pellet: new industrial pre-production plastic nurdles, beached pre-production nurdles, and floating filters, known as biobeads, also retrieved from the environment. Our chemical analyses show that leachates from beached pellets (biobead and nurdle pellets) and highly plasticised industrial pellets (PVC) contain polycyclic aromatic hydrocarbons and polychlorinated biphenyls, which are known to be detrimental to development and other life stages of animals. We also demonstrate that these microplastic leachates elicit severe, consistent and treatment-specific developmental abnormalities in *P. lividus* at embryonic and larval stages. Those embryos exposed to virgin polyethylene leachates with no additives nor environmental contaminants developed normally, suggesting that the abnormalities observed are the result of exposure to either environmentally adsorbed contaminants or pre-existing industrial additives within the polymer matrix. In the light of the chemical contents of the leachates and other characteristics of the plastic particles used, we discuss the phenotypes observed during our study, which include abnormal gastrulation, impaired skeletogenesis, abnormal neurogenesis, redistribution of pigmented cells and embryo radialisation.

1. Introduction

Ubiquitous and persistent, microplastics (plastic particles smaller than 5 mm) have become an emerging contaminant and have been identified as a possible major anthropogenic threat to marine ecosystems, due to the effects they elicit through numerous mechanisms, on multiple life stages and at several levels of the food web (Wright et al., 2013). Primary microplastics are produced at sizes of between 1 and <1000 μm; the main uses are as microbeads in cosmetics and cleaners, pre-production pellets and other small particles such as biobeads. Pre-production pellets, or nurdles, are a key source of primary microplastics (Rochman et al., 2019), entering waterways by the millions annually (Karlsson et al., 2018). Designed to be 3–5 mm in diameter, they are the industrial feedstock for the plastic industry and act as the building blocks for most of the plastic products produced (Mato et al., 2001). Biobeads are high surface area floating filters, 3–4 mm in diameter, designed to aid in filtration in waste water treatment plants by acting as

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attachment substrata for bacterial biofilms (Turner et al., 2019). They are manufactured from plastic recycled from end-of-life waste electrical and electronic equipment, associated with restricted elements such as Br, Cd, Cr, Pb and Sb and found widely contaminating beaches of western Europe (Turner et al., 2019). Secondary microplastics are formed following the photolytic, mechanical and chemical degradation of larger plastic products or fragments.

Plastics are harmful to marine biota by ingestion or entanglement, but they may also serve as vectors for toxic polymer additives and adsorbed environmental pollutants (Teuten et al., 2009). Marine microplastics have been associated to more than 200 organic chemicals (Gauquie et al., 2015). These include both harmful plastic additives originating from the manufacturing process (such phthalates), and environmental pollutants adsorbed from the surrounding seawater such as hydrophobic organic compounds (like polychlorinated biphenyls (PCBs) or polycyclic aromatic hydrocarbons (PAHs)) (Endo et al., 2005; Hirai et al., 2011; Mato et al., 2001; Rios et al., 2007). Exposure of organisms to microplastics and the associated contaminants leaching from the polymer matrix might be an important, but as yet overlooked, uptake route (Hartmann et al., 2017). This leaching could pose a significant threat to marine organisms, since a wide range of plastic additives have been identified as toxic compounds (Lithner et al., 2011) and have been detected leaching from marine plastic (Teuten et al., 2009).

Most marine community structures fundamentally depend on the successful development of planktonic larvae. Mechanical and chemical environmental stressors, such as toxic metal exposure (Itow et al., 1998; Kobayashi and Okamura, 2004), ionizing radiation (Bonaventura and Matranga, 2017; Dahms and Lee, 2010) or anthropogenic activity (Carballeira et al., 2012) can lead to malformations during development. Recent research suggests that plastic and associated leachates may also elicit toxic effects in developing invertebrates, although the evidence has been somewhat equivocal. Virgin plastic pellet exposure has been shown to cause toxic effects on the embryonic development of sea urchins (Martínez-Gómez et al., 2017; Nobre et al., 2015) and mussels (Gandara e Silva et al., 2016). Virgin commercial microspheres have been found to elicit confounding effects, from detrimental to very limited or no impact in sea urchins, sea squirts and other zooplanktonic marine invertebrate larvae, including crustaceans, rotifers and molluscs (Beiras et al., 2018; Cole and Galloway, 2015; Kaposi et al., 2014; Lo and Chan, 2018; Messinetti et al., 2018; Sussarellu et al., 2016; Trifuoggi et al., 2019). Studies suggest that leachates from plastic consumer products cause increased larval mortality and settlement inhibition in barnacles (Li et al., 2016), and developmental arrest and embryonic and larval mortality in pearl oyster (Gardon et al., 2020). Moreover, leachates from coloured plastic products exhibit embryotoxic effects on sea urchin larvae (Oliviero et al., 2019). It is well documented that echinoderm early life stages exhibit a high sensitivity to toxicants, including heavy metals (Hardin et al., 1992; Kobayashi and Okamura, 2004; Pagano et al., 2017) or persistent organic pollutants (POPs) (Anselmo et al., 2011; den Besten et al., 1989; Gunduz et al., 2013; Kobayashi and Okamura, 2002). However, there is little evidence that microplastic can elicit developmental defects without ingestion and instead through leaching associated pollutants into the environmental media, and no thorough description of the abnormalities has been undertaken.

In this study, we report developmental toxicity of microplastic particles leaching into seawater. To determine and compare the toxicity of leachates from different plastics, we cultured embryos of *Paracentrotus lividus* (echinoderm) in leachates of environmental (beached) or new pre-production plastic nurdles, or beached biobeads. We analysed the chemical composition of the water leachates by gas chromatography techniques to study which compounds present could have an effect on embryos and larvae. We expected to find environmental pollutants in the beached samples and industrial additives in the factory samples. We found clear detrimental effects of leachates from plastics that contained chemical substances, and no effect when such substances were not present. We also found that the time of leaching and the concentration of plastic particles in the water are directly correlated with the severity of the phenotypic effects. We present a comprehensive analysis of the developmental abnormalities that occurred after treatment.

2. Materials and methods

2.1. Microplastic particles

Environmental samples: Beach pellets were collected from Tregantle beach, UK, in February 2019. Nurdles and biobeads were manually sorted from other anthropogenic material and organic matter. Biobeads were separated from nurdle pellets based on their 'screw thread' appearance (Turner et al., 2019). Ambiguous particles were discarded.

Industrial samples: New white polyvinyl chloride (PVC) prime plasticised pellets were obtained from Northern Polymers and Plastics Ltd. (UK). Based on previous research and the nature of the polymer, plasticised PVC pellets were chosen as a microplastic with potentially high levels of hazardous additives (Navarro et al., 2010).

Additive-free particles: Virgin (additive-free) low-density polyethylene (PE) granules were obtained from Sigma-Aldrich (cat. N. 428043). The company's product manager was contacted to confirm the absence of additives in the pellets.

2.2. Microplastic composition analysis by fourier-transformed infrared spectroscopy

Sub-samples from each environmental microplastic type (nurdles and biobeads) were subject to attenuated total reflection-Fourier Transform Infra-red Spectroscopy (ATR-FTIR; Agilent Cary 630 FTIR spectrometer; PerkinElmer Spotlight 400 FT-IR Imaging System) to identify polymer composition. FT-IR spectra (near infrared) were obtained for each candidate microplastic piece by scanning in the wave number range between 4000 and 650 cm^{-1}, at a resolution of 4 cm^{-1}, and acquiring a minimum of 4 scans per item. Polymer type was determined by comparing each sample FT-IR spectra against known standard polymer spectra from PerkinElmer's Spectrum software (10.5.3.738).

2.3. Microplastic leachate chemical analysis

60 ml of each plastic type was soaked in 240 ml of artificial seawater (35 PSU, prepared according to Lyman and Fleming (Lyman and Fleming, 1940) (3 replicates) for 72 h at 18 °C in the dark, rocking. Low-density polyethylene pellets were used as a control in every case. Subsequently, the water was filtered through Whatman GF/C glass microfiber filters and subjected to further quantitative and qualitative analyses for selected persistent organic compounds and phthalates. The detailed list of target compounds together with the abbreviations used, as well as particle weights, are provided in the supporting information.

Information regarding the applied analytical procedures and quality assurance are detailed in the supporting information. Briefly, in case of POPs, target compounds were extracted from leachates with hexane according to a modified procedure described by Papale and collaborators (Papale et al., 2017). Clean up and fractionation was performed by absorption chromatography on

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silica gel and aluminium oxide as described by Pazdro (Pazdro, 2008). Hexane was used to extract PCBs and hexachlorobenzene (HCB) (fraction 1) and a mixture of hexane:methylene chloride (9:1 v/v) to extract PAHs (fraction 2). A gas chromatograph was used for qualitative and quantitative analysis of PCBs, HCB and PAHs. An electron capture detector (ECD) was applied for PCBs and HCB detection and a flame ionization detector (FID) for PAHs detection. The identification was checked by the analysis of selected extracts by GC-MS. The concentrations of the analytes in the samples were calculated by using external five-point calibration curves plotted for each compound in the linear range of the detector's response. Phthalates in leachates were analysed according to EPA 8061a method, using solid phase extraction with C18-bonded silica bed and subsequent extraction of target analytes with acetonitrile. Gas chromatography and electron capture detector was used for qualitative and quantitative analysis.

The quantification limits for PCBs in seawater ranged from 0.013 to 0.065 ng/l. The quantification limits for HCB in seawater was set at 3.5 pg/l, and for PAHs from 0.71 to 3.45 ng/l. Quantification limit for individual phthalates was set at 1 μg/l. Recoveries of individual compounds ranged from 77 to 104% depending on the compound. Selected samples were performed in triplicates.

Statistical analysis of the differences between each treatment with the controls was conducted using unpaired *t*-test.

2.4. Leachate preparation for embryo exposure

The concentration of plastic particles used in these experiments was empirically obtained through a series of dilutions (20%, 10%, 4% v/v) and leaching times (24 and 72 h) to determine the concentration of plastic particles needed to elicit effects in sea urchin embryos. Longer leaching times (Suppl. Fig. 1a) and higher concentrations (Suppl. Fig. 1b) produced, as expected, a greater proportion of anomalous embryos and larvae. We subsequently performed experiments treating embryos with water leached for 72 h and at a concentration of 10% for each plastic particle (v/v).

Methods to obtain the microplastic leachate were adapted from Nobre and collaborators (Nobre et al., 2015). In brief, plastic pellets were suspended in filtered seawater (0.22 μm) in 1 l glass bottles at a concentration of 10% (v/v). Pellets were leached for 72 h on a Heidolph orbital platform shaker (Heidolph Unimax 2010), with a continuous shaking at 18 °C in the dark. Leachates were obtained by filtering through Whatman GF/C glass microfiber filters in order to remove particles. Microplastics were removed from direct interaction with the developing embryos to avoid potential mechanical effects of the pellets on the embryos, and to maintain a uniform concentration of leachates during the time of exposure.

2.5. Animal husbandry and embryo exposure

Adult *P. lividus* were housed in circulating seawater aquaria (18 °C) in Napoli (Italy). Gamete release was induced by vigorously shaking ripe animals, followed by an injection of 0.5 M KCL for stubborn adults. Eggs were filtered through 100 μm mesh to remove the jelly coat and debris. Diluted eggs were fertilised in one batch, by adding 5 drops of diluted sperm in single crossings. Fertilisation success was checked to ensure the elevation of the fertilisation membrane in >95% of eggs. Embryos were added to the treatment beakers, containing 500 ml of microplastic leachate or control filtered seawater, at a density of 50 embryos per ml, and left to develop in a static incubator at 18 °C on a 12:12 light dark cycle.

2.6. Index of microplastic impact

To qualitatively asses toxicity of leachate exposure, we classified embryos obtained from each treatment following methodology based on previous studies (Gambardella et al., 2013; Corinaldesi et al., 2017). Live images of a sub-sample of embryos and larvae from each treatment at 24 and 48 h post fertilisation (hpf) were taken in either ZEISS Imager.Z2 or Leica DMI 6000 B microscopes. Embryos and larvae were classified by morphological deviation from the wildtype as by the categories below (Table 1, see supporting information for a more extensive description). We determined the frequency of each category of embryo or larval alteration, and an index of microplastic impact (IMPI) was calculated as follows:

$$IMPI = [0 \times \% \text{ category } 0 + 1 \times \% \text{ category } 1 + 2 \times \% \text{ category } 2 + 3 \times \% \text{ category } 3 + 4 \times \% \text{ category } 4]/100.$$

IMPI gives a continuous range from 0 (no impact) to 4 (all dead). Intermediate levels describe different increasing developmental delays and other morphological malformations as described (Table 1).

Table 1
Index of microplastic impact. Score classification of embryonic (24 hpf) and larval (48 hpf) morphological features.

24 hpf embryo classification	
Score	**Characteristic features**
0	Well-structured, typical archenteron
1	Delayed archenteron formation (lateral view); radialised embryo (vegetal view)
2	No archenteron formed, blastopore and vegetal plate visible
3	Mesenchyme blastula
4	Highly abnormal features; necrosis
48 hpf larva classification	
Score	**Characteristic features**
0	Typical 4-arm pluteus larvae
1	Malformed 4-arm pluteus larvae with crossed or separated tips
2	Reduced body length, stunted arms
3	No arms visible, prismatic shape
4	Pre-larval stages; necrosis

2.7. Immunostaining

Embryos and larvae were fixed in 4% paraformaldehyde in filtered seawater for 15 min at room temperature, washed for 1 min in 100% ice cold methanol and several times in PBST (Phosphate-Buffered-Saline-Tween 0.1%). Samples were incubated overnight in 1 mg/ml BSA and 4% sheep serum (blocking buffer). Next, primary antibodies were diluted in blocking buffer and incubated with embryos for 1.5 h at 37 °C. To mark the serotonergic neurons, we used the rabbit polyclonal Anti-Serotonin antibody (Sigma-Aldrich) diluted 1:1000 in blocking buffer. To label cilia, we used the mouse Anti-acetylated tubulin antibody (Sigma-Aldrich) diluted 1:200 in blocking buffer. To label PMCs and skeletal rods, we used the mouse Anti-Msp130 antibody (1D5) (kindly donated by David R. McClay), diluted 1:5 in blocking buffer. Embryos and larvae were washed multiple times with PBST and incubated for 1 h at room temperature with the secondary antibodies Alexa 555 goat anti-rabbit, Alexa 633 goat anti-rabbit and Alexa 488 goat anti-mouse (Thermo Fisher Scientific) diluted 1:1000 in PBST. After 3–5 washes in PBST, embryos and larvae were mounted with DAPI for imaging with a Zeiss 700 confocal microscope.

3. Results

3.1. Physical characterisation of beached microplastic particles

A sub-sample of randomly selected pellets were analysed by Fourier Transform Infrared Spectrometry to characterise the

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polymer composition of beached pellets. Analysis of nurdles (n = 87) and biobeads (n = 77) revealed the dominant polymer type was polyethylene. For the nurdles, 78% of the pellets were primarily composed of polyethylene, 12% polyamide and 10% polypropylene. For the biobeads, 92% were polyethylene and 8% polyamide.

3.2. Chemical analysis of microplastic water leachates

The microplastics used in this study have been selected on the basis of real-life relevance, using pellets retrieved from the environment (beached pellets), and environmental damaging potential, using new PVC plastic nurdles composed of hazardous monomers and high percentage of additives. Using gas chromatography techniques, we have determined the chemical composition of leachates from each of the plastic particles described in section 2.1: beached biobeads, beached nurdles and PVC, as well as control consisting of water with virgin PE (with no added chemicals). We selected to trace contaminants known to have toxic effects and of environmental relevance and performed an analysis for targeted compounds including phthalates, PCBs, HCB and PAHs (see materials and methods and supporting information for detailed compound list).

In all performed experiments the levels of individual phthalates were lower than the limit of quantification (LOQ) established at 1 ng/ml. Because of the volume of leachate used for phthalate analyses (40 ml), we can assess that less than 8 ng/g plastic was leached from nurdles and biobeads, and less than 7 ng/g for PVC, during the 72 h experiment.

For the analysis of targeted POPs present in the water, we quantified the presence of 7 PCBs, HCB and 16 PAHs (Suppl. Table 1). Individual values were low, sometimes under the quantification limits, but taking the additive concentrations of chemicals, we find a clear increase in all treatment waters in respect with the control (Fig. 1). A recent publication finds comparable concentrations of PCBs, and lower PAHs concentrations, leached from plastic farming gear in French Polynesia (Gardon et al., 2020). In our study, it is clear that leachates from plastic particles collected from the beach (both nurdles and biobeads) were enriched in persistent organic pollutants when compared to control samples. This is in agreement with the general perception that plastics collect persistent organic pollutants from their surrounding environment (Mato et al., 2001). As expected, the concentrations for environmental pollutants in PVC pellets are lower than in beached samples, yet higher than the control.

Taking the sum of the readings for all 7 PCBs targeted, biobead leachates have 24 times, and nurdle leachates 21 times, higher concentration than PE controls. PVC leachates show a lower amount of PCB concentration, equivalent to that of PE controls. HCB was detected only in the case of PVC leachate (Suppl. Table 1), with an increase of nine-fold respect the control.

In the case of the 16 PAHs determined, biobead leachates show the highest concentration of pollutants at 8 times that of the control PE leachates, while nurdles show a 5-fold increase. PVC leachates have a 2.5 times increase when compared to the PE controls.

3.3. Morphological assessment of P. lividus development

To evaluate the toxicity of additives and absorbed pollutants leaching from the microplastics, *P. lividus* embryos were exposed to the leachates of new and beach-collected pellets as described in the Materials and Methods section, and classified according to the degree of morphological alteration and developmental delay (Table 1), to establish an index of severity of the microplastic leachate impact (IMPI).

At 24 h post fertilisation (hpf) (Fig. 2A), most wildtype and PE treated embryos were classified as normal (category 0). Most embryos across biobead and nurdle treatments were classed as delayed (category 1), with an index of microplastic impact (IMPI) of 1.3 for biobead (n = 99) and 1.2 for nurdle (n = 100). Embryos exposed to PVC were classed in a higher category, with most embryos in class 2 and 3, assigning an IMPI of 2.5 (n = 90), accounting for the stronger delay in the development in this treatment (Fig. 2A).

At 48 hpf (Fig. 2B), most larvae of wildtype and PE treated embryos were normal (category 0). Larvae exposed to beached and PVC treatments were classed in categories 2, 3 and 4, referring to a strong phenotypic effect from all leachates (Fig. 2B). Those exposed to PVC leachates (n = 100) had an IMPI score of 2.6, while larvae exposed to the beached microplastic leachates had a score of 2.8 for larvae exposed to nurdle leachates (n = 92) and 2.9 for those exposed to biobead leachates (n = 70).

Our IMPI assigns scores relying on developmental delay and

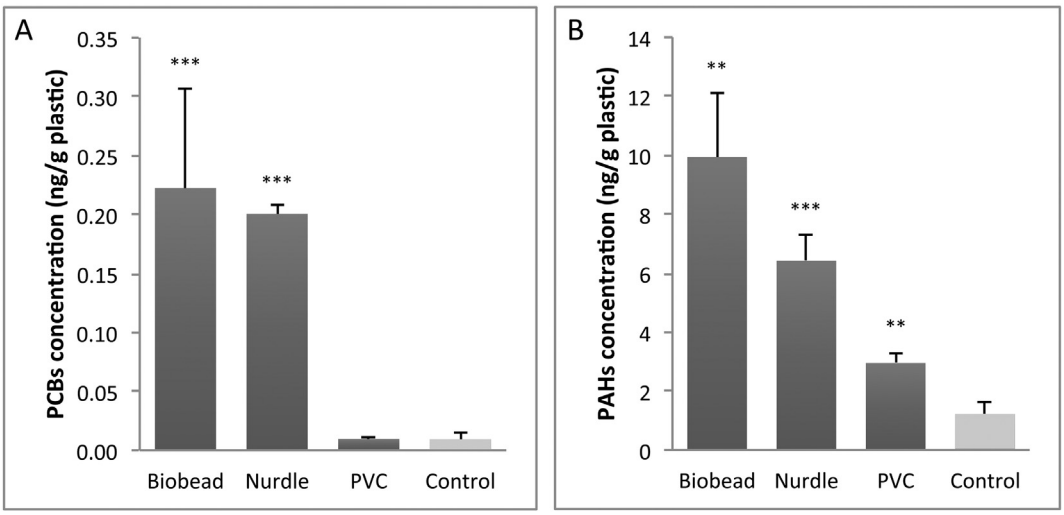

Fig. 1. Concentrations of 7 PCBs (A) and 16 PAHs (B) found in the leachate samples (black) compared to control PE leachates (grey) in ng/g plastic. Individual concentrations are provided in Suppl. Table 1. Asterisks indicate statistically significant differences (**p < 0.01, ***p < 0.001) for the indicated treatment with the control (unpaired *t*-test). Error bars show standard deviations.

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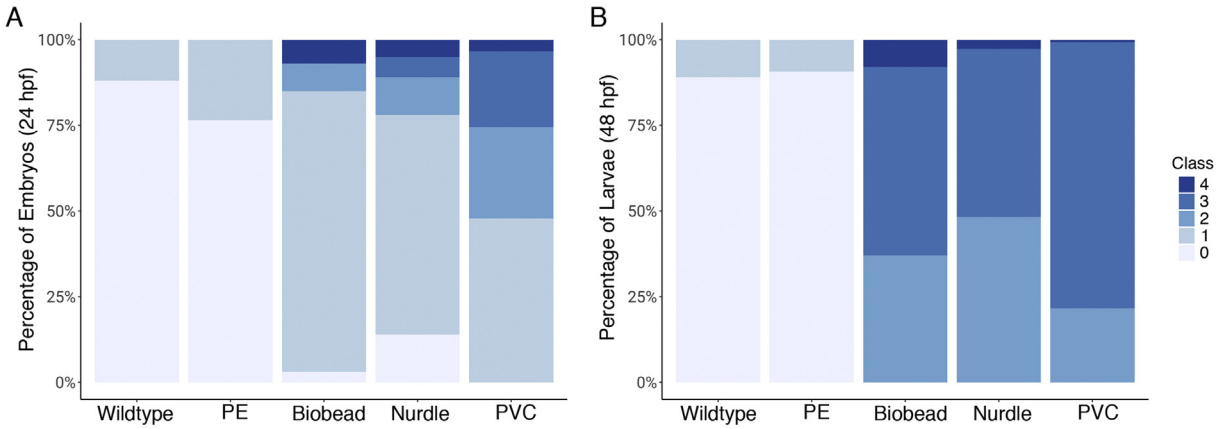

Fig. 2. Effect of beached and industrial microplastic leachates on the development of embryos and larvae of *P. lividus*, with unexposed larva as a control. (A) Percentage of *P. lividus* embryos (24 hpf) classified according to IMPI class for each treatment (B) Percentage of *P. lividus* larvae (48 hpf) classified according to IMPI class for each treatment.

several morphological characteristics. The abnormality seen in any of the categories ranging from 1 to 3 represent strong developmental problems that may lead to unviable embryos or larvae (category 4 already assumes embryo or larval death). This classification allows quantification of the types of abnormalities seen, and is for this reason applied. However, it is important to distinguish between normal (class 0) and abnormal embryos (1–4). Beached and PVC pellets elicited a strong increase in abnormal embryos and larvae, with >86% embryos classed in categories 1–4, across treatments and development stages (24 and 48 hpf; Fig. 2A and B). This percentage of anomalous embryos and larvae, which include both delayed and abnormal phenotypes, in all treatments was significantly higher than controls and PE treated embryos (two proportion z-test, $p < 0.0001$).

Embryos were imaged at 19, 24 and 48 h of development (Fig. 3), which correspond to mesenchyme blastula, late gastrula and early pluteus stages in the wildtype. Severe phenotypic defects were found in embryos exposed to PVC, nurdles and biobeads compared to controls: in general, we found a dose-dependent delay in the development program from 24 hpf, along with phenotypic abnormalities in specific embryonic tissue groups. No differences were observed among treatments at mesenchymal blastula (19 hpf), corresponding to the onset of gastrulation (Fig. 3A, D, G, J, M). At 24 hpf, wildtype embryos show a phenotype corresponding to late gastrulation, with secondary mesenchymal cells populating the tip of a well-formed archenteron elongating towards the animal pole, and primary mesenchymal cells (PMCs) migrating bilaterally from the base of the archenteron (Fig. 3B, B′). At 48 hpf, wildtype larvae show an early 4-arm pluteus with a complete skeleton and well-formed tripartite gut, and the typical distribution of pigmented cells along the body of the larvae (Fig. 3C, C′).

PE leachates treated embryos show no apparent phenotype divergences from the wildtype animals (Fig. 3D, E, F).

Biobead treated embryos generate radialised 24 h gastrulas. The clusters of skeletogenic cells, the PMCs, which are typically located near the blastopore seem to be disorganised in these embryos, presented as a ring of cells around the blastopore and not as bilateral clusters of cells (Suppl. Fig. 2). The overall rounded shape of the embryo suggests a problem in the oral-aboral axis differentiation, coupled with a delay in the elongation of the archenteron as compared to the wildtype (Fig. 3H, H′). The radialised phenotype apparent at 24 h is rescued in pluteus larvae, but a normal phenotype is never achieved. Pluteus larvae of biobead treated embryos are bell-shaped, with short arms. A larger apical organ with longer sensory cilia is very apparent in this treatment, when compared to the wildtype (Suppl. Fig. 3). Anti-acetylated tubulin antibody staining also shows increased number of cilia (Suppl. Fig. 4). We also observe increased pigmented cells (Fig. 3I), which are involved in immune response in sea urchins. These pigmented cells are usually concentrated in the blastocoel around the stomach and the ectoderm at this stage, and not below the apical organ, as we observe in this treatment (Fig. 3I′).

The nurdle treated embryos have an altered and delayed phenotype that is equivalent to the phenotypes seen in the biobead treated samples, but slightly less severe. These also show shorter archenteron, a large number of mesenchyme cells present between the anterior neuroectoderm and the foregut and radialised 24 h gastrulas (Fig. 3K, K′), and abnormal pluteus larvae (Fig. 3L) with a bell-shaped morphology, short arms, increased number and aberrant distribution of pigment cells, and extended apical organs with longer cilia (Suppl. Fig. 3D).

PVC treated embryos had a severe delay in gastrulation by 24 hpf (Fig. 3N), where the vegetal plate had begun to form, mesenchyme cells had ingressed, but there was no primary invagination to form the archenteron, and the blastopore was not clearly formed in most embryos. Misarranged mesenchyme cells could be observed at this time. At 48 hpf, PVC treated pluteus larvae showed a rounded phenotype (Fig. 3O), consistently lacking complete arm and skeletal formation, more typical of a prism stage in the wildtype, at 30 hpf. This delay in development and the morphologically rounded embryos are compatible with a possible reduced development of the spicules, affecting skeletogenesis. Moreover, the number of pigmented cells is reduced. A tripartite gut is seen at this stage, suggesting a normal development of the gut (Fig. 3O).

In summary, we saw treatment specific malformations: the skeleton development seemed impaired in PVC treated larvae, and symmetry, pigment cells distribution and apical organ size in the larvae treated with environmental plastics were abnormal. In the light of these malformations, we performed immunohistochemical staining to observe the effects of leachate contamination on skeletogenic cells (Anti-Msp130 antibody, marking PMCs) and nervous system development (anti-serotonin antibody, marking serotonergic neurons, and anti-acetylated tubulin antibody, marking cilia) (Fig. 4, Suppl. Fig 4)). As observed before, PE treated embryos were equivalent to wildtype embryos (Fig. 4A, B, F, G).

At 24 hpf, PMCs revealed by Anti-Msp130 antibody (Fig. 4A–E, green) show a small delay in skeletogenesis in biobead treated embryos, with shorter longitudinal PMC chains (Fig. 4C). The most affected embryos were, however, PVC treated embryos, where PMCs chains were absent, showing severely impaired skeletal

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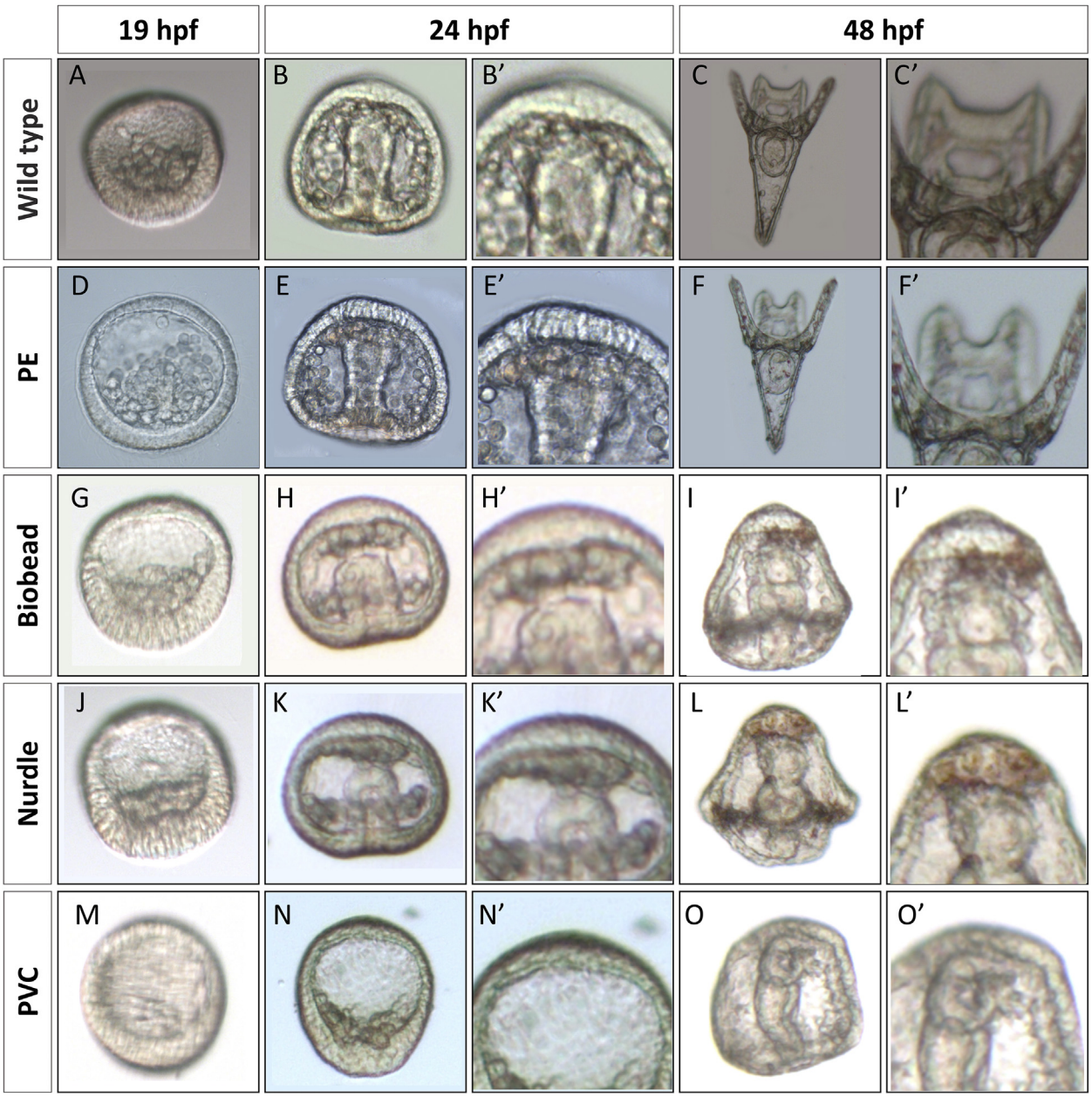

Fig. 3. Phenotypic comparison of wildtype and treatment *P. lividus* embryos and larvae at 19, 24 and 48 h post fertilisation. Treatments plastic leached for 72 h at 10% particle concentrations (v/v). Embryos and larvae shown in H, I, K, L, N, O correspond to severe phenotypes (IMPI class 2 or 3). B′, E′, H′, K′, N′ show close ups of B, E, H, K, N showing the foregut-anterior neuroectoderm and C′, F′, I′, L′, O′ show close ups of the oesophagus-apical organ domain. See text for details.

morphogenesis (Fig. 4E). At 48 hpf we observed abnormal but complete skeleton in nurdle treated embryos (Fig. 4I, green), with a small malformation in the posterior arms in biobead treated embryos (Fig. 4H, green). In PVC treated embryos the impairment of skeletogenesis was still evident at this stage, as no skeletal rods had yet been formed, although PMCs were able to fuse (Fig. 4J, green).

Apical organ development was assessed by staining serotonergic neurons (anti-serotonin antibody, Fig. 4F–J, F′-J′, magenta), since these are the neurons found within the apical organ. Wildtype and PE treated embryos had equivalent serotonergic structures (Fig. 4F, F′, G, G′, magenta). Biobead treated larvae showed an increase in the number of serotonergic neurons and this change reflected mostly a rise in the number of interneurons (Fig. 4H, H′, magenta). Nurdle treated pluteus displayed decreased in number of serotonergic neurons (Fig. 4I, I′, magenta), and PVC treated larvae showed complete absence of immunoreactivity (Fig. 4J, J′, magenta).

4. Discussion

We have shown that plastic leachates in seawater contain chemicals which are known to be detrimental to animals. We have also demonstrated that microplastic leachates from beached pellets (biobead and nurdle pellets) and highly plasticised industrial pellets (PVC) elicit severe, consistent and treatment-specific developmental abnormalities in sea urchin (*P. lividus*) embryos. In contrast, embryos exposed to virgin polyethylene leachates, with no additives nor environmental contaminants, exhibited normal

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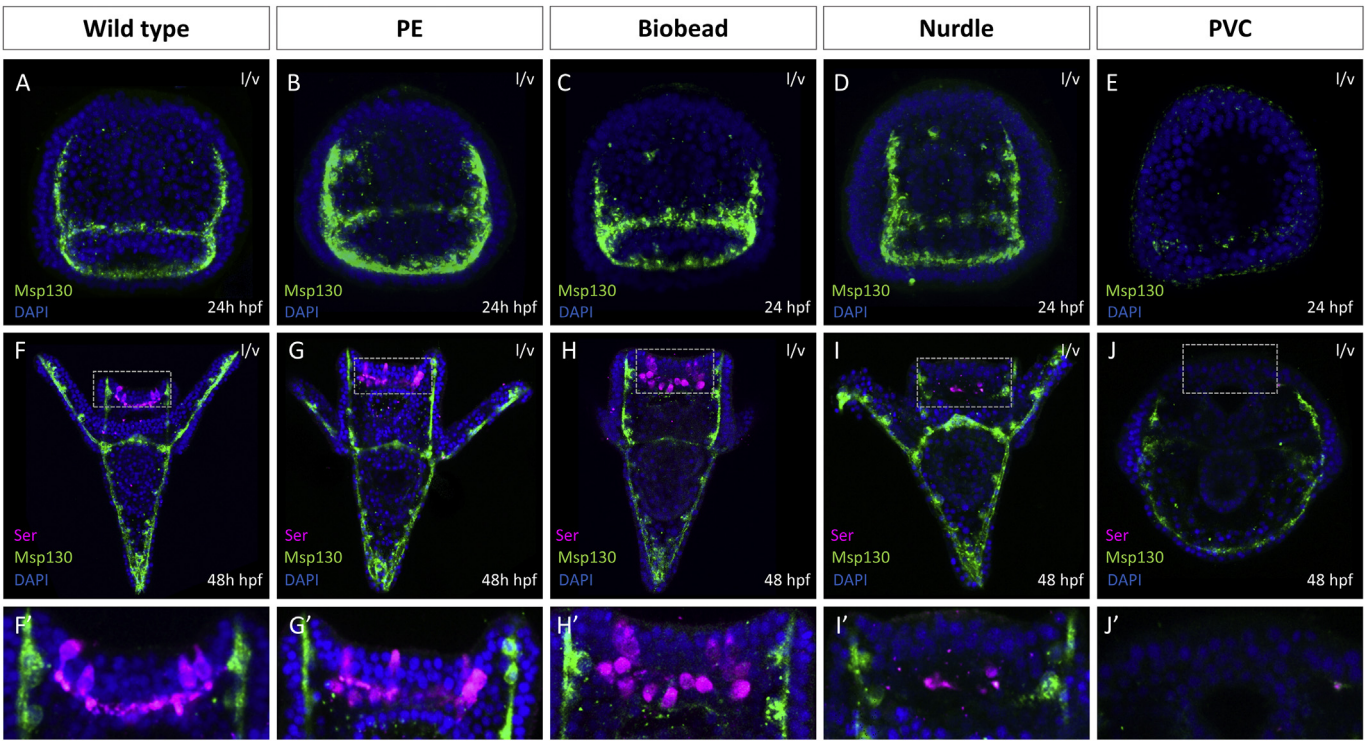

Fig. 4. Immunostaining with Anti-Msp130 (skeletogenesis, green), anti-serotonin (magenta) antibodies in 24 (panels A–E) and 48 (panels F–J) hpf embryos for the different treatments. Insets in panels F–J enlarged in F'-J'. Nuclei stained in blue by DAPI. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

development. Hence, our results strongly suggest that the abnormalities observed are a result of both the environmental adsorbed contaminants and pre-existing industrial additives within the polymer matrix.

We screened for a series of possible contaminants—additives from the manufacturing process and environmental pollutants adsorbed from the seawater—leaching from the plastic particles: phthalates, PCBs and PAHs. As common, harmful, industrial additive in plastic production, we analysed phthalate contents. We predicted higher levels of phthalates in our industrial PVC samples (Jimenez et al., 2001; Stringer et al., 2000). For their environmental ubiquity and known toxicity, we targeted PCBs and PAHs. We expected increased content of these compounds in beached samples (Gorman et al., 2019; Rochman et al., 2013). We detected increased levels of PCBs and PAHs in all three of our model plastics, except PCBs in PVC leachates, but we were not able to quantify phthalates in our water samples. Lack of phthalates detection could be explained due to the conservative levels of detection used in our approach, or due to the longer leaching time required for phthalates to migrate to seawater (Paluselli et al., 2019). We detected the highest concentrations of environmental pollutants in biobead leachates compared to nurdles for both PCBs and PAHs. Biobeads are buoyant filtration substrates, with ridged edges to increase surface area to facilitate bacterial growth. Since both types of beached pellets were collected at the same location, it is likely that the increased surface area aids pollutant adsorption onto the biobeads. We also see more severe phenotypic defects in the biobead treated sea urchins than in the nurdles, which conform with the chemical results. As expected, the lowest increases in PAHs were in PVC leachates. Here we expected very low levels of PCBs, as they are banned substances according to the Stockholm convention. Environmental pollutants could have been adsorbed from the atmosphere to the PVC particles in storage or transport. However, manufacturing PVC uses large quantities of additives such as phthalates, Bisphenol A and flame retardants (Markarian, 2007), and we expected to find high levels of phthalates in PVC samples, which could explain some of the developmental abnormalities described. The limits of quantification in our study did not allow us to detect these compounds, but in a non-targeted analysis using solid phase microextraction, where the suggested compounds were based on the NIST reference library, we found phthalates leached from PVC pellets after three days (see supporting information). In fact, no organic extracts obtained from the water samples were clear, suggesting that many other organic compounds could have been released from plastics. In future research, non-targeted as well as targeted analysis should be carried out to gain a better representation of the chemicals present in the water samples.

Our study uses plastic particles obtained from the environment, as well as pre-production nurdles destined to plastic factories. In contrast, previous work on the effects of plastic particles on the development of different sea urchin species have used plastic microspheres designed for laboratory experiments, with equivocal results. When using polyethylene microspheres, two teams found no effects on the survival or development of two species of sea urchin, including *P. lividus* (Beiras et al., 2018; Kaposi et al., 2014), and work using polystyrene microspheres (Messinetti et al., 2018) showed no difference in the survival rate but significant differences in body and arm length of plutei reared at different microplastic concentrations, but with changes in different directions depending on the concentrations of plastics. All these microspheres were known to have no chemicals from polymers able to leach into the water. Using polystyrene microspheres of unknown additive content, another study found significantly increased numbers of malformations, cytogenetic aberrations and developmental arrest at blastula-gastrula stage (Trifuoggi et al., 2019). When using microspheres with additives or residual and toxic monomers (Martínez-

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Gómez et al., 2017), researchers found toxicity in developing embryos, and specifically more from the leachates than from the exposure to plastics directly in the water. However, the use of microspheres might be deemed unrealistic to the types of plastic currently found as pollutants in nature. Using fragments of new plastic toys Oliviero and collaborators (Oliviero et al., 2019) reported formation of exogastrulas and abnormal larval length. This work linked the toxicity observed to chemicals present in the microplastics, in particular heavy metals, but no study of the leachates was reported. An earlier study reported malformations after exposure to factory and beached nurdles (Nobre et al., 2015), with no further description of the phenotypes. In our study, however, we were able to characterise in detail malformations of specific tissues and disruption to particular developmental processes.

We find the severity of the defects to be directly correlated to the concentration of plastics in the water and time of leaching: the more plastic particles and the longer the time of leaching, the stronger the phenotypical aberrations in embryos and larvae (Suppl. Fig. 1). This concentration response has been reported before in a study exposing embryos to micronized plastic particles (Oliviero et al., 2019). Other studies using plastic leachates from microspheres show stronger effects at the lower concentration treatments (Martínez-Gómez et al., 2017), but higher concentrations of small particles may cause microplastic aggregation, which in turn decreases the actual concentration of plastics. We used particles that are at the higher end of the spectrum of the microplastic sizes, and aggregation is negligible in our case. Other variables influencing the concentration and nature of the chemicals in the beached samples include the origin of the particle (pre-production nurdle or biobead), the time spent in the water, the locations visited by the particle and the exposure to UV and other environmental conditions (Mato et al., 2001; Ogata et al., 2009; Rochman et al., 2019). We identify clear dose and treatment-dependent morphological defects in developmental processes, that we next discuss in light of the chemical contents of the leachates and the characteristics of the particles used.

Oral-aboral axis malformation and embryo radialisation

Adult echinoderms are radially symmetrical animals, but their larvae develop in a bilateral fashion. Wildtype sea urchin eggs are polarised along a single maternal axis, the animal-vegetal axis. Soon after fertilisation, a secondary axis develops, the oral-aboral axis, which defines the axis running from the mouth region to the opposite side. Correct establishment of the oral-aboral axis is key to the bilateral development of sea urchin embryos. In our study, we find oral-aboral axis differentiation delay and embryo radialisation for embryos exposed to beached microplastics leachates. Wildtype gastrulating embryos show two bilateral clusters of PMCs at the vegetal pole (Decker and Lennarz, 1988). In radialised embryos, PMCs are known to form a ring in the lateral ectoderm (Duboc et al., 2004; Hardin et al., 1992) and we observe a similar phenotype in biobead and nurdle treated embryos, with radially distributed PMCs on the vegetal pole of the gastrula, surrounding the archenteron (Suppl. Fig. 2). In the PVC treatment this phenotype is less evident, and is likely to be confounded by the severe developmental delay.

We have demonstrated the existence of PAHs in leachates from all our plastic treatments. Exposure to PAH results in disruption of axial development and patterning in the sea urchin *Lytechinus anemesis* (Pillai et al., 2003) and in zebrafish (Fairbairn et al., 2012). However, radialised embryos, with PMCs formed a ring in the lateral ectoderm, are also obtained from exposure to heavy metals (Hardin et al., 1992). In addition to the potential for PAH to affect the radialisation of the embryo, the stronger phenotypes observed in the biobead leachates could be explained by their high content of metals (Turner et al., 2019), which can also leach in the waters and are known to affect axis formation (Hardin et al., 1992). At later stages, the radialisation is partially rescued in the larvae of all treatments, although the larvae in our beached samples display a bell shape morphology similar to that described in radialised larvae by Duboc or Hardin and collaborators (Duboc et al., 2004; Hardin et al., 1992).

Impaired skeletogenesis and reduced arm growth

The skeleton of sea urchin larvae forms a structure which is under very tight developmental control, and disruption of this process can lead to an abnormal skeleton, abnormal shape of the larvae and failure to develop further. All larvae exposed to the leachates from PVC, biobeads and nurdles experience defects in skeletogenesis, with lack of skeletal formation most evident in those embryos exposed to PVC leachates. Our immunostaining of PMCs via Anti-Msp130 antibody confirms the severe delay of skeletogenesis in the gastrula of PVC and, to a lesser extent, biobead and nurdle leachates treated embryos. The skeleton grows from a biomineral structure (spicules), which is secreted by the PMCs in the sea urchin gastrula. If symmetric PMC clusters do not form properly around the blastopore, spiculogenesis will not be initiated (Decker and Lennarz, 1988), and skeletal formation will be affected. We do not observe the expected clusters of PMCs in all treated gastrulas (Suppl. Fig 2). The skeletogenic problems may therefore be a result of abnormal early spiculogenesis caused by the mispositioning of PMCs.

Previous research has reported similar skeletal malformations such as reduced arm growth or lack of skeletogenesis as a result of exposure to PCBs and PAHs. In zebrafish embryos, high concentrations of Aroclor1254 (PCB) resulted in skeletal morphological deformities (Ju et al., 2012). In sea urchins, *Hemicentrotus pulcherrimus* treated with two different PAHs showed reduced or supressed spicule formation (Suzuki et al., 2015), and *Psammechinus miliaris* exposed to PCBs and PAHs developed shorter arms (Anselmo et al., 2011). Skeletal malformations have also been linked to heavy metal exposure, like Zn (Kobayashi and Okamura, 2004) or Ni (Hardin et al., 1992). Hardin et al. (1992) suggest that nickel chloride exposure alters commitment of ectodermal cells along the dorsoventral axis. Hence, our results are both consistent with PAH and PCB contamination or with putative heavy metal exposure, although other substances not targeted by our analysis may also be involved.

Altered number and redistribution of pigmented cells

Pigmented cells in *P. lividus* have the role of immune cells within the larval ectoderm and gut, providing protection from microbial colonisation and invasion. Upon infection, pigmented cells normally embedded in the aboral ectoderm of the larva, change shape from dendritic to spherical and migrate in the blastocoel to surround the stomach of the larva (Buckley and Rast, 2017). PVC leachate treatments show a reduction of the number of pigmented cells, probably a result of the strong reduction of mesoderm derivatives as seen in the case of skeletogenesis in this treatment. However, we observe an increase in number of pigmented cells inside the blastocoel in the beached treatments, suggesting an additional stressor within those treatments. In *P. lividus*, PCBs are known to stimulate the cellular response of reactive oxygen species production (Coteur et al., 2001). In our study, we have found a higher concentration of certain PCBs in beached particles, consistent with finding a higher number of immune cells in the larvae of

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these treatments. However, the surface of microplastics acts as an ideal habitat to harbour microbial communities (Zettler et al., 2013), and could be a reservoir for pathogenic microbes (Keswani et al., 2016). Biobead pellets themselves are actively used in waste water treatment plants to form a biofilm of bacteria and aid in treating contaminated water (Turner et al., 2019). Therefore, the increase in cells with immune function, particularly around the stomach, in the pluteus larvae exposed to beached pellets could also be the result of increased microbial invasion from the biofilm on the beached pellets.

Abnormal serotonergic neuron connections and larger apical organ

P. lividus sensory serotonergic neurons in the apical organ are involved in modulating muscular and ciliary activity (Bisgrove and Burke, 1986). Our environmental and PVC plastic leachates treated embryos show clear neurogenic defects. In PVC treated embryos, serotonergic neurons are completely absent, and in nurdle treated larvae, these neurons are almost completely lost and dislocated. Paradoxically, in biobead treated larvae serotonergic neurons, and especially the interneurons, are increased in numbers. Previous work in bivalves has showed that exposure to PCB leads to a dose-dependent decrease in serotonergic neurons (Smith et al., 1999) and decrease in serotonin production and in the size of the serotonin-synthesizing region (Kreiling et al., 2000). It is also known that exposure to PAH in developing zebrafish and rat cell lines cause neurotoxicity, and that this is concentration dependant, even if the singular component of the mixed samples has no toxic effect (Geier et al., 2018; Tang et al., 2003). Despite the low concentration of PAHs and PCBs found in our water samples, this additive effect of different compounds could explain the serotonergic toxicity seen in our experiments. However, it is unclear what is causing the increase in serotonergic neurons seen in the biobead treatments, but this may be related to decoupling of the signals that restrict neuronal fate in the apical organ or alternative effects of the non-described compounds leaching from the particles.

The apical organ is a tufted cilia sensory structure common in invertebrate larvae within which the serotonergic neurons are contained. The beached pellet treatments show larger apical organs, as well as longer and more numerous cilia in that area (Suppl. Fig. 3), which may be related to the changes in neural structures we observed. However, in laboratory genetically induced radialised embryos (Duboc et al., 2004), longer cilia are also observed, suggesting the genetic mechanisms that generate both phenotypes could be in place.

In most cases, plastic fragments will be found at lower concentrations in the sea than used in our study. However, plastic particles may become trapped at high concentrations in spaces such as rock pools, ports, aquaculture farms, very polluted shores, or can be floating *en-mass* after spill events. In addition, microplastics are also increasingly being described in terrestrial and freshwater habitats (Allen et al., 2019; Dris et al., 2016; Li et al., 2020) and environments with high plastic concentrations, such as landfills, may have similar leaching mechanisms, carrying pollutants downstream with the potential to affect fresh water and land animal development. Some studies have suggested that the ingestion of plastic particles represents a limited threat to marine larvae (Kaposi et al., 2014), while others suggest that pelagic invertebrate embryos exposed to microplastics in confined environments may experience lethal toxic effects due to leached chemicals from plastic materials (Martínez-Gómez et al., 2017), and that *P. lividus* accumulate some PCBs more efficiently when exposed via water than food (Danis et al., 2005). Our data show that chemicals are indeed leached from beached and industrial pellets, and that they have deleterious effects in development. The concentrations of the chemicals in the pellets will depend on many factors, including the original load in the pellets (such as in our PVC samples) or their history in the sea (Ogata et al., 2009). It is also important to be aware of the possible degradation of some chemicals by UV and other environmental factors, which will help lower the toxic effect on development, although one should note that PAH toxicity could also be enhanced by UV radiation (Huang et al., 1993). Bioaccumulation of a large number of POPs in *P. lividus* adults has been reported (Rocha et al., 2018) thus demonstrating that even local and temporally low concentrations of toxic chemicals may cause greater problems in time. Our findings demonstrate that such chemicals leach from plastic particles in the environment, even if at small concentrations, and that these produce strong developmental abnormalities in sea urchins, and point to clear and specific detrimental effects of plastic pollution on animal development.

5. Conclusions

Our results show that beached and industrial plastic particles can leach persistent organic pollutant (PAHs and PCBs) into seawater, and that sea urchin embryos (*P. lividus*) in these waters will develop abnormally, probably resulting in non-viable larvae. The toxicity of the water is directly dependent on the concentration of plastic particles and the duration of leaching. We find that beached preproduction nurdles and biobead pellets have a deleterious effect on sea urchin development, as do industrial PVC nurdles. These developmental abnormalities, which are treatment specific, include developmental delay, malformation of skeletal structures and nervous and immune systems, as well as abnormal axis formation. Although we find that PAHs and PCBs are leached into the water, those are at low concentrations. We believe that other potentially toxic chemicals, such as phthalates and metals, have a role in the developmental abnormalities we observe in the sea urchins. A non-target analysis for a wider range of organic compounds, as well as analysis of metals released into the water, would shed light on the key toxicants released.

Author statement

Flora Rendell-Bhatti: Methodology, Conceptualization, Formal analysis, Investigation, Visualization, Writing – Early draft; **Periklis Paganos:** Investigation, Formal analysis, Visualization, Writing - Review & Editing; **Anna Pouch:** Investigation; **Christopher Mitchell:** Investigation, Methodology, Resources; **Salvatore D'Aniello:** Resources, Writing - Review & Editing; **Brendan J Godley:** Methodology, Funding acquisition, Writing - Review & Editing; **Ksenia Pazdro:** Methodology, Formal analysis, Resources, Writing - Review & Editing; **Maria Ina Arnone:** Methodology, Formal analysis, Resources, Writing - Review & Editing; **Eva Jimenez-Guri:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115744.

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